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Luiten, Paulus; de Jong, Giena; SCHUURMAN, T

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# Cerebrovascular, Neuronal, and Behavioral Effects of Long-Term $\text{Ca}^{2+}$ Channel Blockade in Aging Normotensive and Hypertensive Rat Strains

P. G. M. LUITEN,<sup>a,b</sup> G. I. DE JONG,<sup>a</sup> AND  
T. SCHUURMAN<sup>c</sup>

<sup>a</sup>*Department of Animal Physiology  
University of Groningen  
Kerklaan 30, 9751 NN Haren, the Netherlands*

<sup>c</sup>*Institute for Neurobiology  
Troponwerke, Neurather Ring 1  
5000 Cologne 80, Germany*

In the search for mechanisms underlying the detrimental influence of the aging process on the functions of the nervous system, the progressive derangement of intracellular calcium homeostasis has gained increasing attention.<sup>1-3</sup> Changes in calcium homeostasis during aging are considered to lead to a gradual but chronic  $\text{Ca}^{2+}$  overload with serious consequences for neuronal excitation and cellular communication.<sup>2,4</sup>

In contrast to extensive knowledge on altered  $[\text{Ca}^{2+}]$  in the cellular and extracellular components of the cardiovascular system during aging,<sup>5</sup> such information on cerebral vasculature is scarce. The total free  $[\text{Ca}^{2+}]$  in major cardiac and body arteries show a dramatic, progressive increase in an age-dependent fashion, which is accelerated by risk factors like nicotine, diabetes, and hypertension.<sup>6</sup>

Like the peripheral vessels, however, the vasculature of the brain is prone to considerable pathological changes during the aging process,<sup>7,8</sup> which are apt to exert a profound influence on the neuronal functions of the brain.<sup>9</sup> Because the blood-brain barrier (BBB) limits free entry of nutrients and metabolites from the blood stream to the parenchyma, the condition and integrity of the cerebral microvascular system may have a profound impact on general neuronal activity and the nervous control of behavioral functions.<sup>10</sup> As such, the causal mechanism of behavioral impairment as a consequence of aging may be regarded as being one of the key questions in aging research. The importance of the BBB for brain functioning prompted us to study the consequences of the aging process with specific interest in the relation between vascular, neuronal, and behavioral parameters.

<sup>b</sup> Address for correspondence: Dr. P. G. M. Luiten, Department of Animal Physiology, University of Groningen, Kerklaan 30, 9751 NN Haren, the Netherlands.

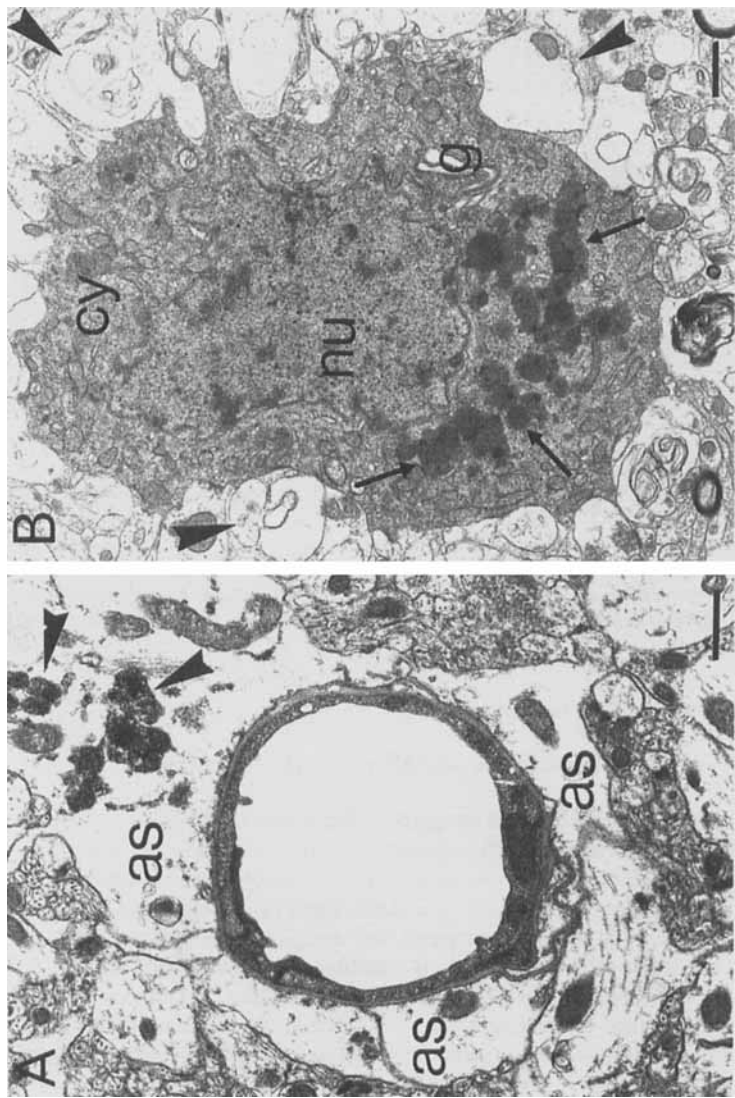
In the homeostatic regulation of intracellular free calcium  $[Ca^{2+}]_i$ , various mechanisms are involved that interact in a complex fashion. Free intracellular  $Ca^{2+}$  is the result of a balance between receptor-, channel-, and electric potential related  $Ca^{2+}$  influxes, intracellular release mechanisms, buffering by  $Ca^{2+}$  binding proteins, intracellular uptake and storage, and efflux by ion exchange and ion pumps.<sup>11</sup>

In the search for tools to antagonize the chronic elevation of  $[Ca^{2+}]_i$  associated with brain aging, it has become a promising strategy to limit  $Ca^{2+}$  influx by blockade of  $Ca^{2+}$  entry pathways.<sup>12</sup> Nimodipine, a lipophilic dihydropyridine acting as an L-type channel  $Ca^{2+}$  antagonist, easily crosses the membranes of the BBB, and this way has ready access to intra- and extracellular components of neuronal, glial, and vascular domains of the nervous system. Evidence has accumulated in recent years that blockade of  $Ca^{2+}$  influx via L-type channels with this compound exerts powerful effects on neuronal,<sup>4</sup> cerebrovascular,<sup>13</sup> and behavioral<sup>14</sup> alterations in aging mammals. Nimodipine was demonstrated to enhance neuronal excitability and improve behavioral and cognitive performance in senescence in several mammalian species including man.<sup>15,16</sup> These nimodipine findings together with our own experience with drug application on cerebrovascular condition prompted us to investigate effects of chronic application of this drug on some vascular, neuronal, and behavioral characteristics of aging normotensive rats.

The nimodipine effects in normotensive animals were extended with studying the influence of this drug in aging hypertensive stroke-prone rats. Hypertension is considered a pathological condition that during aging poses an additional risk factor for the development of cardiovascular and cerebrovascular infarctions and ischemia.<sup>17</sup> In that sense hypertension may be thought to accelerate the dysfunctions and pathologies attributed to the aging process. Hypertension enhances the already dramatic aging-related increase of  $[Ca^{2+}]_i$  in the vascular tissue in several vessels of the peripheral arterial system, pointing to a common mechanism in disturbance of  $Ca^{2+}$  homeostasis. Hypertension not only affects peripheral vessels, but also threatens cerebral vasculature in senescence when stroke and edema may develop.<sup>7,17-19</sup> The fact that hypertension accelerates  $[Ca^{2+}]_i$  in the vascular wall in the course of the aging process suggests that aging and hypertension share common mechanisms of altered  $Ca^{2+}$  homeostasis. For that reason it became interesting to establish the influence of the  $Ca^{2+}$  entry blocker nimodipine in aging hypertensive rats.

## EFFECTS OF NIMODIPINE IN AGING NORMOTENSIVE RATS

When investigated by electronmicroscopy, a large variety of alterations can be observed in the brain of the aging mammal. In senescence changes occur in essentially all cellular and extracellular components of the brain such as nerve cells, various glial cell types, and cerebrovascular cells. As described by many investigators, these changes include the gradual accumulation of lipofuscin (Fig. 1A), excessive myelin breakdown by oligodendrocytes, neuronal degeneration (Fig. 1B), synaptic reorganization and glial hypertrophy, and degenerative changes of the vascular wall. Notably the latter category of vascular breakdown was studied in greater detail with specific interest for the progressive course of events and the influence of chronic treatment with nimodipine on this vascular degenerative process.

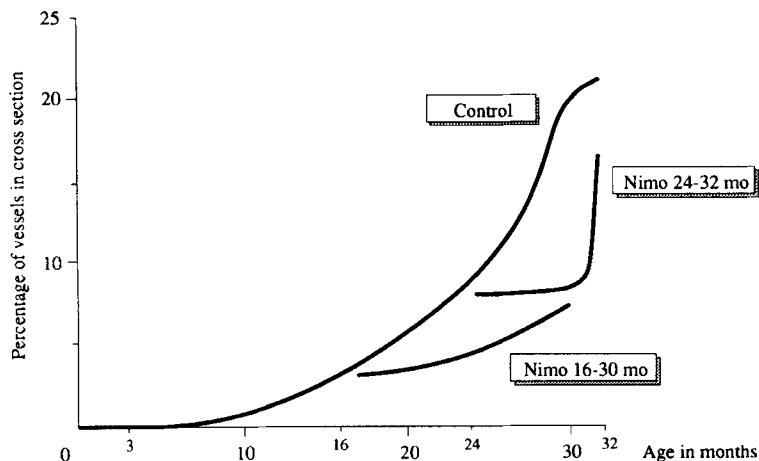


**FIGURE 1.** (A) Electron micrograph of hypertrophied astroglial endfeet (as) surrounding a microvessel in the cortex of a 30-month-old rat. Arrowheads point to lipofuscin granules, a common feature of aging glia and neurons. (B) Degenerating cell body of a pyramidal neuron in the cortex characterized by the irregular shape of cell and nucleus. The cytoplasm is electron dense and contains many lysosomes (arrows). Scale bar = 1  $\mu$ m.

**FIGURE 2.** Cortex of rat aged 30 months. **(A)** Appearance of perivascular deposits consisting of a dense amount of banded collagen fibrils (c). The vascular lumen is lined by the endothelium (e) containing several large vacuoles, but with an intact tight junction. Astrocytes are hypertrophied (as). **(B)** Thickening of basement membrane (\*) as a form of perivascular deposition. Scale bar = 1  $\mu\text{m}$ .

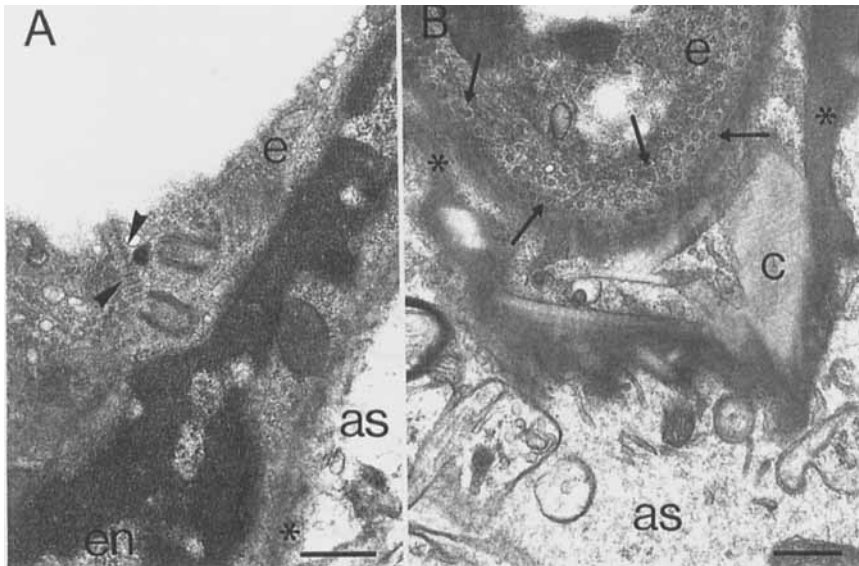
### *Aging, Nimodipine, and Microvascular Morphology*

This study was focused on the integrity of the microvessels as they comprise the anatomical substrate of the BBB. As reported in a sequence of papers, several structural alterations occur in senescence such as stages of degeneration of pericytes<sup>20</sup> and a series of infrequent anomalies and depositions of collagen-like and collagen-derived components in the microvascular wall designated as fibrosis and basement membrane thickening<sup>7,8,21</sup> (FIG. 2). It is notably the perivascular deposit, which, throughout the lifespan of the Wistar rat of approximately 32 months, is subject to a prominent influence of treatment with the calcium blocker nimodipine. In aging control animals the incidence of perivascular deposits gradually increases up to the age of 30 months and then levels off over 30 months in the parietal motor cortex.<sup>13</sup> During the latter final life stage the nature of the deposits changes from an obvious collagen fiber appearance to an amorphous bed of perivascular basement membrane thickening most likely a result of molecular depolymerization.<sup>13</sup> During the aging process we treated groups of Wistar rats chronically with an optimal dose of 1,000 ppm nimodipine administered via their daily food intake. The drug effects were studied after several treatment periods from 16-30 months, 24-30 months, and 24-



**FIGURE 3.** Diagram with the development of perivascular deposits during the lifespan of Wistar rats up to the age of 32 months. Note that the gradual increase during aging levels off at ages over 30 months. Treatment with the  $\text{Ca}^{2+}$  antagonist nimodipine from 16-30 months suppresses the formation of the deposits, as it does from 24-30 months. Over the age of 30 months drug treatment becomes ineffective.

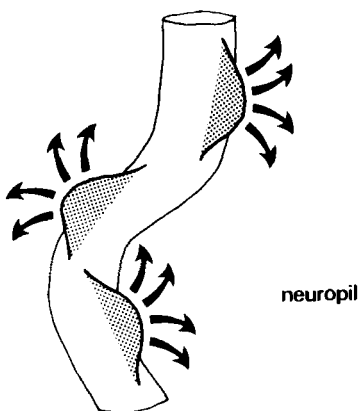
32 months.<sup>13,22</sup> After all treatment periods the incidence of perivascular deposits was significantly suppressed except for the final life phase over 30 months. Here we observed that further treatment could no longer delay the malformations of the perivascular wall (FIG. 3). The treatment effects were proportional to the duration of treatment and the starting point of nimodipine application. More detailed analysis of the deposits in the microvascular wall revealed the collagen fibrotic nature of the perivascular malformation, which is characterized by typical banded fibrils with a periodicity of 64 nm. An important observation from a functional point of view was the apparent endothelial origin of the fibrosis<sup>13</sup> (FIG. 4A). In several instances collagen-like fibrils could be detected within the endothelial vacuoles or cytoplasm, whereas in the region with the thickened basement membrane often large amounts of pinocytotic vesicles occurred indicative of abnormal transport processes over the BBB (FIG. 4B). At the same time the affected microvessel was surrounded by enlarged astrocytic endfeet. The complex of aberrant microvascular structure may well be interpreted as the morphologic basis of dysfunctions of the BBB that coincide with the decreased BBB transport capacity during aging<sup>23-25</sup> (FIG. 5). The aforementioned observations were made on the parietal cortex as a representative of forebrain cortical structures. Sample studies on hippocampus and spinal cord indicate that the aging-related vascular decline and the nimodipine effects were basically similar in these structures of the central nervous system. The spinal effects, however, were of a lower magnitude, which may be related to the lower density of L-type  $\text{Ca}^{2+}$  channels in the brainstem and spinal cord.<sup>26</sup>



**FIGURE 4.** (A) Short collagen fibril fragment (*arrowhead*) in the cytoplasm of the endothelial cell (e). (B) Occurrence of large number of pinocytotic vesicles (*arrows*) in the endothelial cell of a microvessel in the cortex of an aged (30-month-old) rat. Within the basement membrane the collagen fibrils are deposited, while the astrocytic endfeet are enlarged. Scale bar = 0.5  $\mu\text{m}$ .

### *Aging, Nimodipine, and Synaptic Ultrastructure*

Apart from the vascular changes in aging animals, a variety of neuronal changes also were recorded. In more detail we established the impact of the calcium antagonist



**FIGURE 5.** Schematic impression of local perivascular deposits in microvessel walls in the aging brain. The impact of microvascular aberrations is indicated by the *arrows* that represent dysfunction of the blood-brain barrier and its influence on the surrounding neuropil.

on the synaptic density and size exemplified on the supragranular layer of the dentate gyrus. This region was selected because of the general consensus that in this region the number of synapses is decreased in aged mammals including man in health and disease.<sup>27-29</sup> The synaptic changes during aging were assessed with the ethanolic-phosphotungstic acid method which selectively stains for synaptic structures for electron microscopic application. Our observations were in line with the reports of others that the synaptic density expressed as the number of synapses per cubic millimeter significantly decreased by a fourth.<sup>30</sup> Although the average synaptic surface did not change, the decrease in density coincided with a very significant decline in total synaptic surface area. The effect of nimodipine treatment became prominent in both density and total synaptic surface parameters, such that the decline in density was entirely prevented by the drug, while the synaptic surface was significantly higher than that of the untreated age-matched controls. In summary, these nimodipine effects may be interpreted as a long-term counteractive effect on the age-dependent decline of the synaptic structure in this part of the hippocampus.

### *Aging, Nimodipine, and Calcium Binding Proteins*

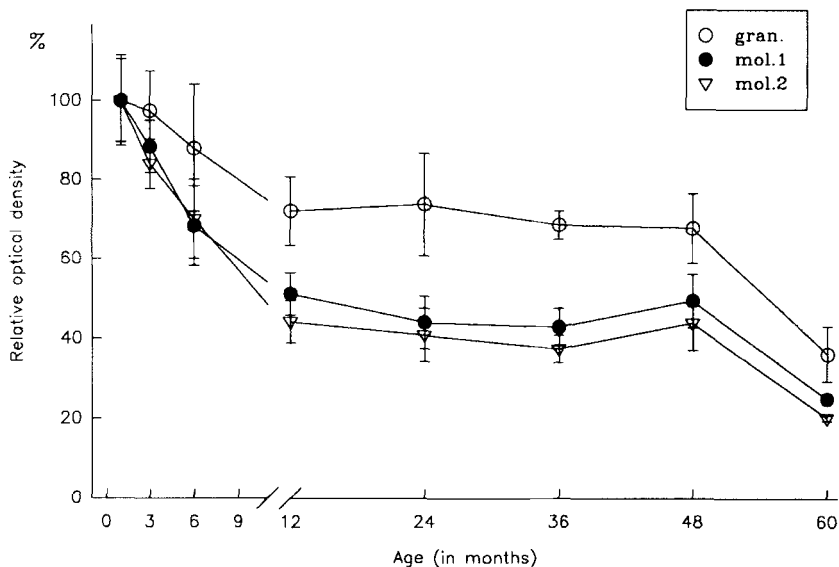
Inasmuch as the currently used calcium antagonist exerts such a profound influence on neuronal structure and function, we included some calcium-related parameters of aging neurons. As one parameter that is of potential importance for calcium homeostatic mechanisms, we investigated the immunoreactivity (ir) of calcium-bound forms of calcium binding proteins during aging in rabbit and rat.<sup>31</sup> In rabbit dentate gyrus the calbindin-D28k (CaB) protein showed a striking decline during the first 12 months of the rabbit's lifespan after which the CaB-ir level remained constant up to the age of 48 months (Fig. 6). Between 48 and 60 months a second phase of CaB decline was observed when CaB-ir reached a level of about 25% of the optical density of 1-month-old animals. This age-dependent decrease of CaB-ir was essentially similar for granule cells, their dendrites in the dentate molecular layer, and the mossy fiber projection to the CA3 region. A similar trend was found for CaB-positive cells in the CA1 pyramidal cell layer, but not in the stratum oriens. In the rabbit, age had no effect on the numbers of parvalbumin-positive GABAergic neurons in the various regions of the cornu ammonis.

In the rat, CaB-ir strongly declined in senescence to a similar degree as in rabbit notably in the dentate gyrus and the neocortex (Fig. 7), but not in the CA1 of the hippocampus. As in the rabbit the number of parvalbumin-positive cells did not change in cortex and hippocampus. In rat we also investigated if the CaB and PARV pattern changes were influenced by chronic nimodipine treatment. Chronic treatment with the calcium antagonist only moderately antagonized the decline of the two calcium binding proteins investigated. Only in the cortex was the reduction of CaB attenuated, and this was significant in the parietal cortex (Fig. 7).

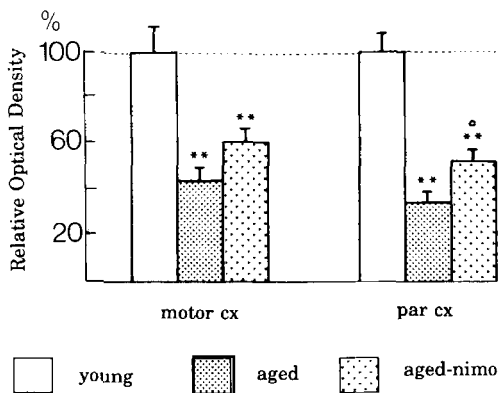
### *Aging, Nimodipine, and Open-Field Behavior*

There are numerous reports on the impact of calcium channel blockers on various behavioral parameters. (See the contributions of Traber and Fannelli, this volume.)

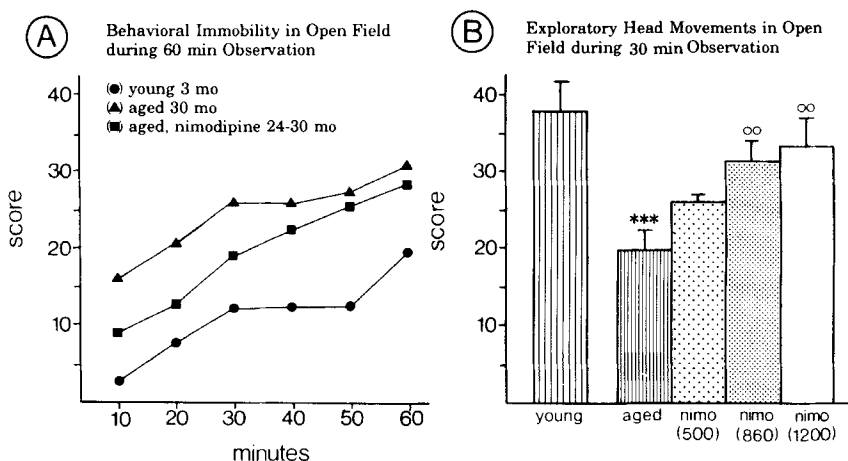




**FIGURE 6.** Calbindin D28k immunoreactivity (CaB-ir) in the various layers (granule cell layer, gran.; inner molecular layer, mol.1; outer molecular layer, mol.2) of the dentate gyrus at eight different ages in the rabbit. Immunoreactivity was quantified with image analysis. Values were calculated as a percentage of the highest value of 1-month-old rabbit, which was set at 100%. The CaB-ir rapidly declines during the first 12 months, stabilizes up to 48 months, and is followed by a final reduction in extremely aged cases of 60 months.



**FIGURE 7.** CaB immunoreactivity measured by optical density image analysis and expressed as percentages in layers II-IV of parietal and motor cortex in young (3-month-old), aged (30-month-old), and aged (24-30-month-old), nimodipine-treated Wistar rats. The sharp decrease in CaB-ir in the aged rat is slightly attenuated by nimodipine treatment, which was significant in the parietal cortex (\*\* $p < 0.01$ ; \* $p < 0.05$ ).



**FIGURE 8.** (A) Immobility scores measured during 60-minute observation of rats exposed to novelty-induced behavioral arousal in an open field test. Young animals show a lower level of immobility than do aged animals. The immobility of the nimodipine group (24–30 months of treatment) was significantly lower than that of the age-matched controls. (B) The reversed effects are seen by scores of head movements as a measure of exploratory behavior in the open field. Nimodipine effects are dose-dependent.

In short, these studies demonstrate that chronic nimodipine application improves behavioral scores in motor performance and spatial memory, facilitates and accelerates learning and conditioning, improves short-term memory in the radial maze, and attenuates the age-related decline in visual discrimination.<sup>32,33</sup> Also the first trials in humans with various types of dementia point to improved neuropsychological scores and a prophylactic effect on the progression of the dementing process.<sup>15,34</sup>

In the same animals used to investigate cerebrovascular and neuronal effects, we also assessed the behavioral scores in a novelty-induced behavioral arousal paradigm (Fig. 8). In this test the spontaneous behavior of young (3 months), aged (30 months), and aged, nimodipine-treated rats (24–30 months) was recorded during 60 minutes. Aged animals of 30 months compared to 3-month-old controls were characterized by a very significant suppression of exploration, rearing, walking, and sniffing accompanied by an equally large increase in immobility. All these age-dependent spontaneous behavioral expressions were significantly antagonized in a dose-dependent manner by chronic nimodipine application starting at the age of 24 months<sup>16</sup> (Fig. 8B). The observed behavioral effects of the aging process (as in the small open field test in aging) and the impact of nimodipine both corroborate the effects of this drug in motor and cognitive behaviors as reported by others in various species.<sup>14,15,32–34</sup>

### *Summary of Normotensive Brain Aging*

Aging in the rodent, as in other mammalian species, reveals a wide variety of cerebrovascular, neuronal, and behavioral changes that collectively represent a slow

but gradual deterioration in the cellular components of the brain, its blood supply, and BBB, leading to deficiency of basically all behavioral performance. In a series of investigations we demonstrated a significant effect of the L-type calcium channel blocker nimodipine on a number of microvascular, synaptic, and behavioral parameters. Control over calcium influx eventually will yield maintenance of microvascular function, control of the endothelium over BBB function, improvement of astrocytic and glial support, and delay of neuronal breakdown and neuronal signal transduction. The mechanism by which a compound such as nimodipine affects the aging process as yet remains a subject of study. Convincing evidence exists that aging affects several aspects of calcium-regulating homeostasis. More recent studies establish the notion that aging burdens the cell with a persistent overload of basal  $[Ca]_i$  levels and delays the mechanisms that restore intracellular calcium concentrations.<sup>35,36</sup> Convincing electrophysiological reports<sup>2,4</sup> indicate that a compound such as nimodipine attenuates the prolonged afterhyperpolarization in aging hippocampal pyramidal neurons, a phenomenon that is associated with improved behavioral conditioning by nimodipine.<sup>4</sup>

The way in which nimodipine exerts such a powerful effect in delaying the deterioration of the microvascular wall during aging remains unclear. The aberrant microvessels in the aging rat brain are often circumvented by hypertrophied astrocytes. Because astrocytes play an important role in the maintenance of the BBB, these observations suggest that a loss of cerebrovascular integrity induces BBB alterations. More specifically, the increased number of pinocytotic vesicles in the endothelial cytoplasm in compromised microvessels points to disturbed nutrient transport over the microvascular wall. Such impaired BBB transport function has been demonstrated in the aging CNS<sup>10,23,24,37</sup> and clearly affects the surrounding neuropil and subsequent neuronal functioning.

On first sight, the percentage of microvessels displaying microvascular deposits may seem relatively small. However, each microvessel probably will contain several anomalies along its longitudinal surface, so that examination of microvascular cross-sections provides the relative density of these aberrations. Assuming that each microvessel in the aging brain displays loss of integrity and concomitant impaired nutrient transport along its surface, the devastating implications for neuronal functioning become clear (FIG. 5). In this light we can consider the protective effect of chronic calcium channel blockade (50% fewer microvessels with deposits in animals aged up to 30 months) as highly beneficial. Moreover, the fact that a calcium antagonist can be so effective implicates that disturbed calcium homeostasis of the microvascular wall strongly contributes to aging-related impaired neuronal functioning and consequent behavioral performance.

Most evidence points to the endothelial cell as the main origin of microvascular deterioration during aging. Not only are the number of endothelial pinocytotic vesicles increased in the aging brain, but also small collagen fibrils are present within the endothelial cytoplasm of aberrant microvessels. The cerebrovascular effects of calcium antagonists were thus far explained by vasodilatory influences on larger vessels, but never anticipated the presence of L-type calcium channels in endothelial cells. In contrast to those in the peripheral circulation, the larger arteries in the CNS are major determinants of local microvascular pressure.<sup>38</sup> Our studies cannot exclude that the beneficial influence of calcium channel blockade on microvascular integrity in the aging brain is secondary to alterations in the larger resistance vessels. When

larger blood vessels in the brain dilate under the influence of nimodipine, the microvascular blood pressure will be reduced, possibly preventing the formation of microvascular aberrations. However, all the ultrastructural evidence for endothelial involvement in microvascular deterioration during aging and the influence of calcium channel blockade lead to an alternative hypothesis. Although most studies indicate that  $\text{Ca}^{2+}$  enters endothelial cells through nonselective cation channels,<sup>39,40</sup> Bossu and coworkers<sup>41</sup> demonstrated the presence of L-type calcium channels in isolated capillary endothelial cells. We now hypothesize that increased calcium influx through L-type calcium channels in the endothelial cell contributes to the declined microvascular condition and subsequent impaired neuronal functioning in the aging CNS (Fig. 12).

## EFFECTS OF NIMODIPINE ON AGING SPONTANEOUSLY HYPERTENSIVE STROKE-PRONE RATS

### *General Condition and Occurrence of Stroke*

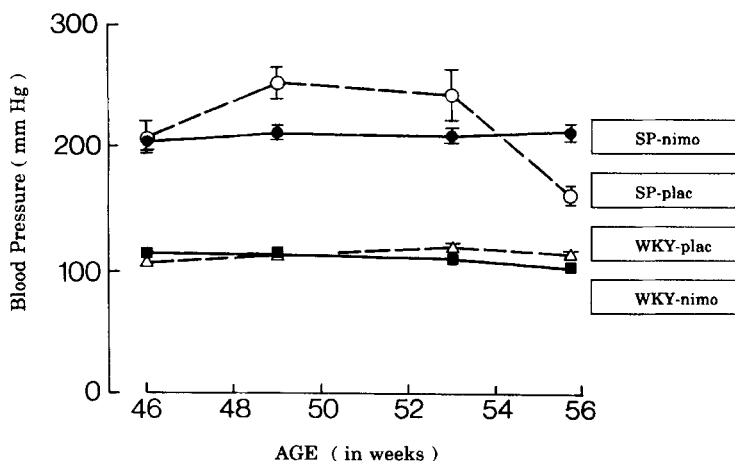
High blood pressure is now commonly regarded as the most frequently occurring risk factor in vascular pathology in the peripheral circulation. Analysis of coronary and larger arterial tissue reveals high blood pressure as a major contributor of  $\text{Ca}^{2+}$  accumulation in the vascular wall notably combined with aging.<sup>6</sup> Little is known about the influence of elevated blood pressure and aging on  $\text{Ca}^{2+}$  contents in the cerebral vasculature. On the other hand, there is well documented evidence for the acute effects of the Ca antagonist nimodipine on the vasodilatory characteristics of the cerebral vasculature,<sup>42</sup> which is probably mediated via the smooth muscle cells of the larger vessels. Here we investigated the impact of the  $\text{Ca}^{2+}$  entry blocker in aging spontaneously hypertensive stroke-prone (SHR-SP) rats, in which the effects of chronic drug application were assessed on a number of vascular, physiological, neuronal, and behavioral parameters. The influence of high blood pressure on the aging process became readily obvious as the hypertensive strains showed all signs of aging-related deterioration in body condition and behavioral performance at relatively very early stages of life as compared to normotensive Wistar-Kyoto (WKY) animals. In a first set of experiments SHR-SP rats were chronically treated with nimodipine applied via daily food intake at a concentration of 1,000 ppm starting at the age of 46 weeks (immediately before the period of stroke development) up to the age of 56 weeks. During the treatment period the physical condition (fur condition and locomotor activity) gradually deteriorated. The body weight of the placebo-treated SHR-SP animals decreased from 330 to 280 g, whereas the nimodipine-treated group (SP-nimo) remained at a consistently higher level and even showed a slight increase from 323 g to an average of 354 g. No effects of treatment were found in age-matched normotensive WKY rats (TABLE 1). Furthermore, during the 10-week treatment all SP-placebo rats developed neurologic (irritability and paralysis) and histologic signs of stroke, but none of the SP-nimo cases did. In fact, because of the increasing number of lethal SP-placebo cases, the treatment period had to be terminated to maintain a sufficient number of placebo cases for statistical analysis. All of the 11 SP-nimo animals survived the experimental period, whereas 4 of 11 SP-placebo rats died with the symptoms of stroke. The incidence of stroke at the end

**TABLE 1.** Effect of Treatment on the Occurrence of Neurologic and Histologic Signs of Stroke, Body Weight, and Brain Weight in Spontaneously Hypertensive Stroke-Prone (SHR-RP) Animals at the Start of the Experiment (Onset) and after 10 Weeks of Treatment with Nimodipine Food (1,000 ppm) or Placebo Food

Group	Age (wk)	Rats with Stroke	Body Weight (g)	Brain Weight (g)
SHR-SP onset (5)	46	2	330 $\pm$ 15.6	2.04 $\pm$ 0.20
SHR-SP nimo (7)	56	0	354 $\pm$ 7.1**	1.81 $\pm$ 0.02
SHR-SP plac (7)	56	7	281 $\pm$ 9.3*	2.53 $\pm$ 0.11**

NOTE: Significantly different from onset group at \* $p < 0.05$ ; \*\*  $p < 0.001$ .

of the experimental period was accompanied by the presence of severe brain edema in the SP-placebo rats. The edema became apparent from the heavily swollen brain immediately after removal from the skull and was quantitatively expressed by the significant increase by 40% in brain weight in the SP-placebo versus the SP-nimo animals (TABLE 1), whereas the body weight of the SP-placebo group significantly decreased. It is important to note that the prevention of stroke by nimodipine could not be attributed to a blood pressure decrease by the drug. Nimodipine had no effect on the very high pressure of the SHR-SP animals and even appeared to stabilize the blood pressure at a value of 220 mm Hg (FIG. 9).



**FIGURE 9.** Development of blood pressure (BP) in spontaneously hypertensive stroke-prone (SHR-SP) and control Wistar Kyoto (WKY) rats treated with nimodipine or placebo food. In neither the WKY nor the SHR-SP group did nimodipine lower BP values. In the SHR-SP cases nimodipine appeared to stabilize the high BP levels.

*Stroke, Hippocampal Alterations, and Behavioral Dysfunction*

A major finding in the first series of experiments with SHR-SP animals was the neurochemical changes that occurred in the hippocampal cell groups in animals that had neocortical strokes. Apparently as a neuropathological consequence of cortical strokes, hippocampal pyramidal neurons revealed abnormally enhanced immunoreactivity for protein kinase  $C\gamma$ , whereas the GABA synthesizing enzyme GAD and its colocalized calcium binding protein parvalbumin in the GABAergic interneurons were significantly decreased.<sup>19</sup> Taken together, these stroke-induced alterations were interpreted as GABAergic cell degeneration leading to abnormal disinhibition of the pyramidal cell group. The lack of inhibition or chronic overstimulation of the pyramidal cells then would yield an abnormally high level of PKC $\gamma$ -ir, probably mediated by relatively high activation of G-protein-coupled receptor types. These changes in the neurochemical nature of hippocampal cell groups did not occur in the aging SHR-SP animals that were treated for 10 weeks with nimodipine and that were free of strokes. The patterns of PKC $\gamma$ , parvalbumin, and GAD immunoreactivity of the SP-nimo animals were basically similar to those in the control WKY rats.

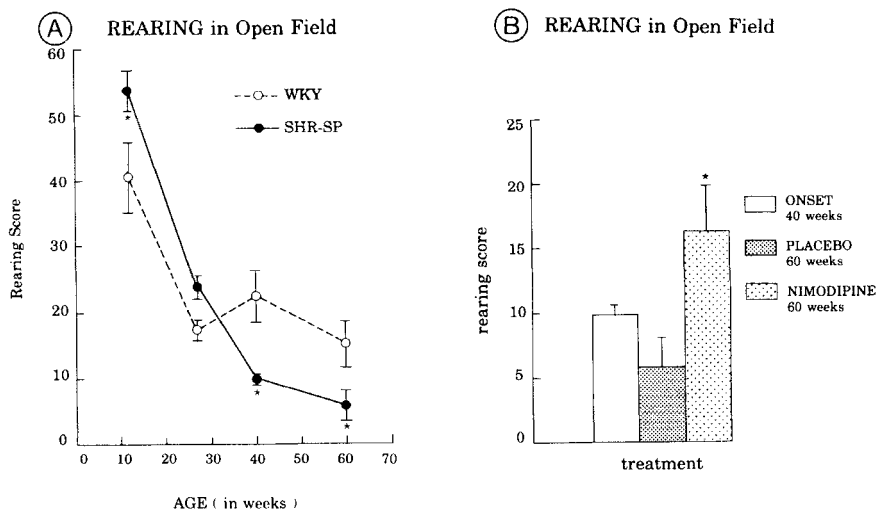
Before sacrifice the SP-nimo and SP-placebo animals were tested for their novelty-induced behavioral arousal in an open-field task. In this test condition the time the animals spent on exploration of their new environment was recorded and revealed an almost threefold higher level of activity of the SP-nimo group than of the stroke victims of the SP-placebo group.<sup>19</sup> It may therefore be concluded that the occurrence of stroke and the concurrent pathological condition of the hippocampal circuitry in these animals coincides with near total absence of behavioral arousal.

It remained to be determined, however, if maintenance of the normal hippocampal and behavioral features in the SP-nimo cases was the result of prevention of stroke by nimodipine or a direct neuronal effect of nimodipine in aging hypertensive conditions. In other words, what is the behavioral profile and the condition of hippocampal neuronal parameters in aging SP rats before the development of stroke. For these reasons we performed a second series of experiments in which WKY and SHR-SP rats were tested and treated with the calcium antagonist nimodipine. Animals of the second experiment revealed a somewhat lower blood pressure level of around 180 mm Hg (220 mm Hg in the first experiment) in which the symptoms of stroke started to appear at the age of 50 weeks (compared to 42 weeks in the first experimental group). Nimodipine was administered from 40-60 weeks to both WKY normotensive controls and SHR-SP rats. During this treatment period none of the WKY animals and SP-nimo rats died, whereas only 50% of the animals in the placebo SHR-SP survived (TABLE 2). None of the remaining SP-placebo rats showed neurological or histological signs of cerebrovascular strokes. This is also reflected by the similar brain weights of all SHR-SP groups and thus by the lack of brain edema formation (TABLE 2). Also the general condition and body weight of the SP-placebo rats did not decline during the observation period. As in this first experiment nimodipine did not diminish blood-pressure in normotensive WKY and hypertensive SHR-SP rats (TABLE 2).

In the experimental setup we followed the behavioral scores in the open field test over the animals' lifespan from 12-60 weeks. It was previously shown by others that SHR rats are more active in a novel environment than are their normotensive

TABLE 2. Effects of Nimodipine Treatment on the Percentage of Animals that Survive, Blood Pressure, Body Weight, and Brain Weight in Wistar Kyoto (WKY) and Spontaneously Hypertensive Stroke-Prone (SHR-SP) Animals at the Start of the Experiment (Onset) and after 20 Weeks of Treatment with Placebo Food or Nimodipine Food (1,000 ppm)

Group	Age (wk)	Survival	Rats with Stroke	Blood Pressure (mm Hg)	Body Weight (g)	Brain Weight (g)
WKY onset (6)	40	—	0	100 ± 4	428 ± 5	2.07 ± 0.03
WKY nimo (6)	60	100%	0	106 ± 3	426 ± 11	2.15 ± 0.03
WKY plac (6)	60	100%	0	111 ± 4	415 ± 6	2.19 ± 0.02
SHR-SP onset (6)	40	—	0	161 ± 7	368 ± 7	1.70 ± 0.03
SHR-SP nimo (6)	60	100%	0	184 ± 2	378 ± 4	1.96 ± 0.01
SHR-SP plac (8)	60	50%	0	179 ± 11	359 ± 3	1.90 ± 0.02

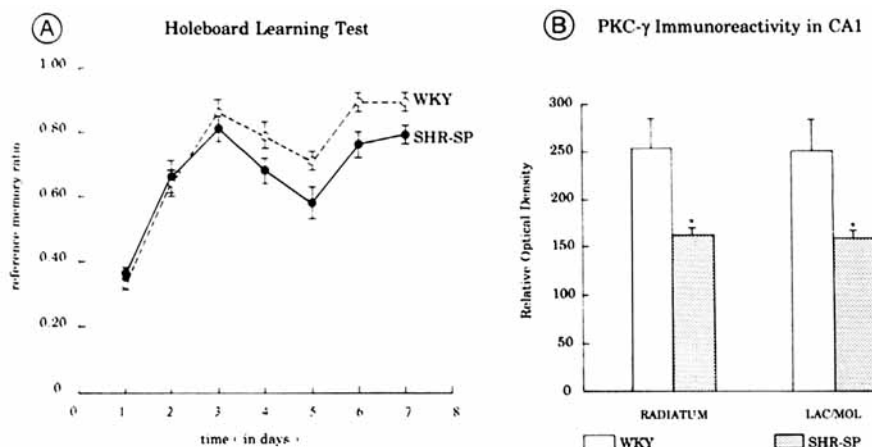


**FIGURE 10.** (A) Behavioral scores of novelty-induced exploration activity in an open field in the lifespan of SHR-SP animals compared with age-matched WKY controls. The initial hyperarousal and hyperactivity expressed by rearing of the animals are reversed to an almost lack of activity in the accelerated aging period of the SHR-SP group. (B) SHR-SP rats treated with nimodipine from 40–60 weeks show a significantly higher level of novelty-induced arousal than do the SHR-SP animals receiving placebo.

controls,<sup>43,44</sup> which corroborates our data in which young SHR-SP rats (12 weeks of age) displayed significantly more rearing behavior than did WKY controls (Fig. 10A). It is generally accepted that especially rearing as a component of exploratory behavior declines rapidly with advancing age. Despite the higher rearing scores at young age, the older SHR-SP animals (40 and 60 weeks of age) show significantly less rearing behavior in the open field than do WKY controls (Fig. 10A). These data indicate that aging-related behavioral alterations occur earlier in genetically hypertensive rats. Nimodipine treatment from 40–60 weeks of age increased the rearing scores, even when compared to that in animals aged 40 weeks (Fig. 10B). In other words, nimodipine completely counteracted the accelerated behavioral decline in aging hypertensive rats before they developed neocortical strokes.

In a recent pilot experiment we compared the performance of older nonsymptomatic SHR-SP rats (40 weeks) with age-matched WKY rats in a holeboard spatial orientation learning test that requires an intact hippocampus. The holeboard contains 16 equidistant holes in which rats were trained to learn a pattern of four baited out of 16 holes, after which the reference memory ratio (RMR) was calculated. At the age of 40 weeks the RMR of SHR-SP rats was significantly lower than that of WKY controls (Fig. 11A). The latter demonstrates that the spatial orientation capacity of aging hypertensive animals is reduced compared to that of their normotensive controls. Similar findings were described by Wyss *et al.*<sup>45</sup> who showed early impairment of cognitive functioning in SHR rats.





**FIGURE 11.** Performance of SHR-SP and age-matched WKY rats at the age of 40 weeks in the holeboard spatial learning test. In this test the animals had to learn a random spatial pattern of 4 baited holes of a total of 16 holes. Reference memory scores (visits food holes + revisits food holes/total visits food holes and non-food holes) of the WKY rats was significantly higher (MANOVA,  $p < 0.05$ ) than those of the SHR-SP group. **(B)** Twenty-four hours after completion of the holeboard test, learning task-induced PKC $\gamma$  immunoreactivity (PKC-ir) enhancement in the hippocampal CA1 area was quantified by optical density measurement. The PKC-ir response was significantly higher in the WKY than in the SHR-SP animals, consistent with their higher level of performance in the holeboard.

Previous work from our group<sup>46</sup> revealed that the immunoreactivity (ir) for PKC $\gamma$  was enhanced in columns of the hippocampal formation of mice and rats that underwent the holeboard task. This increased PKC $\gamma$ -ir was most prominent in the dendritic fields of the CA1 area and can be attributed to increased activation of CA1 pyramidal cells.<sup>46</sup> We examined PKC $\gamma$ -ir in the hippocampus CA1 of both SHR-SP and WKY animals after holeboard training by image analysis. The relative optical density was measured in the CA1 stratum radiatum and in the lacunosum moleculare of all trained animals. In both areas the relative optical density of PKC $\gamma$ -ir was significantly higher in the WKY animals (Fig. 11B). This suggests a relation between learning performance in the holeboard task and PKC $\gamma$ -ir in the CA1, because the better learners (WKY animals) display a denser PKC $\gamma$ -ir in the dendritic fields of the CA1. However, it remains to be established if the difference in PKC $\gamma$ -ir is related to learning performance or strain differences. However, preliminary data show that PKC $\gamma$ -ir does not differ between naive WKY and SHR-SP rats at 12 weeks of age (data not shown), ruling out a general strain difference. Other preliminary data show that PKC $\gamma$ -ir remains stable throughout the age of 12–40 weeks in WKY rats, and declines in none-stroke SHR-SP rats. The latter suggests that decreased activation of CA1 pyramidal cells, as reflected by a reduced level of PKC $\gamma$ -ir, in aging nonsymptomatic SHR-SP rats underlies the impaired learning performance of these animals.

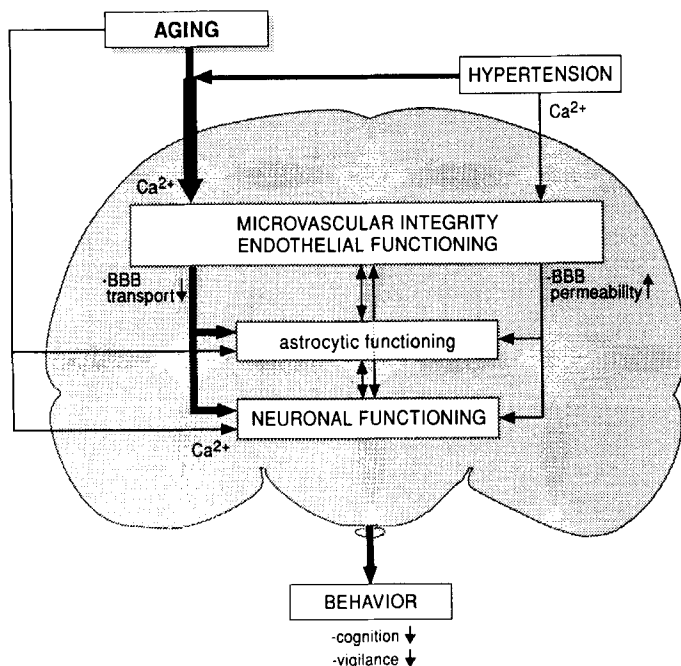
## SUMMARY AND CONCLUSIONS

The pathogenesis of essential hypertension is not fully understood, but most of the cardiovascular, metabolic, neurogenic, and humoral abnormalities are explained by dysfunctions in the control of intracellular  $\text{Ca}^{2+}$  concentrations in the cells of the vascular wall.<sup>47,48</sup> Most theories of disturbed calcium regulation focus on the calcium concentration within vascular smooth muscle cells.<sup>47</sup> The implications of hypertension for the increased calcium content of aging arteries seem to be clear, but were only studied in the peripheral circulation; hypertension prominently augments the aging-related accumulation of calcium in the vessel wall.<sup>5</sup>

Although the contribution of calcium overload in hypertensive cerebrovascular damage is well documented,<sup>42</sup> it is not clear yet if hypertension per se is the main cause of hypertension-associated calcium-dependent cerebral damage. Thus far, the hypotensive effects of most calcium antagonists were extensively described, and their efficacy in stroke prevention was proven.<sup>19,42</sup> Earlier studies indicated that chronic administration of nimodipine revealed a protective effect in the occurrence of strokes in SHR-SP rats, yielding a decreased mortality rate.<sup>42</sup> Because nimodipine did not lower the extremely high blood pressure of these animals,<sup>19,42</sup> the mechanisms behind such nimodipine-induced stroke prevention may be attributed to a direct cerebrovascular and/or neuronal action of nimodipine.

Hypertension is generally considered a vascular pathologic condition, and most research has been directed towards the influences of hypertension on large peripheral arteries such as the aorta and coronary artery. The influence of the CNS on the regulation of cardiovascular system and blood pressure regulation was described in detail, and the role of the CNS in hypertension also was the subject of study.<sup>45</sup> The increased risk of stroke in hypertensive subjects generated numerous studies on the precise nature of compromised cerebrovascular functioning under hypertensive conditions.

Few data are available on  $\text{Ca}^{2+}$  alterations in cerebral neurons during hypertension. Honda *et al.*<sup>49</sup> demonstrated that voltage-dependent  $\text{Ca}^{2+}$  uptake was higher in cortical synaptosomes from SHR than from normotensive animals and suggested that an important alteration in  $\text{Ca}^{2+}$  channel characteristics may occur in SHR brain synaptosomes. Although the density of L-type calcium channels was shown to be higher in the hippocampus of SHR rats,<sup>50</sup> others reported that the number of L-type calcium channels was significantly lower in the brain of SHR rats than WKY normotensive controls.<sup>51</sup> The latter data suggest that hypertension may be associated with similar alterations in neuronal calcium homeostasis as demonstrated for aging in normotensive subjects.<sup>50,52</sup> To date little is known about the relationship between hypertension, the cerebrovascular condition, and neuronal functioning in the CNS. A major finding in these and other studies is that the hypertensive condition strongly accelerates the neuronal and behavioral alterations commonly associated with senescence in normotensive animals. The accelerated behavioral decline of spontaneous hypertensive rats<sup>45,53</sup> indicates that hypertension also progressively affects neuronal functioning during aging. We anticipate that hypertension combined with aging will have a profound impact on the structure of the vascular wall of both larger and fine vessels in the brain. As such, hypertension in aging animals may considerably contribute to impaired neuronal functioning in such animals. We hypothesize that endothelial cell



**FIGURE 12.** Survey diagram depicting the influence of the aging process and of a hypertensive condition on microvascular integrity and function of the endothelial lining of the microvascular wall. Aging and hypertension apparently mediated by  $\text{Ca}^{2+}$ -dependent mechanisms directly affect the vascular condition and functioning of the blood-brain barrier. This way aging may indirectly influence glial and neuronal functioning. The aging process naturally exerts direct  $\text{Ca}^{2+}$ -mediated effects on the glial and neuronal condition as well. Together aging and hypertension have a profound impact on all behavioral functions regulated by the nervous system.

dysfunction (due to disturbed calcium homeostasis) is a major causal factor for the accelerated decline in CNS functioning in aging hypertensive subjects (FIG. 12). This way the efficacy of the calcium channel blocker nimodipine in stroke prevention can possibly be explained by maintenance of endothelial integrity and condition in aging hypertensive rats. The current results also allow the conclusion that maintenance of calcium balance, without reduction of blood pressure, is a fruitful strategy in therapeutic treatment of essential hypertension as a risk factor for brain dysfunction and stroke.

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